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Dual functions of the nucleus-encoded factor TDA1 in trapping and translation activation of *atpA* transcripts in *Chlamydomonas reinhardtii* chloroplasts

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SUMMARY

After endosymbiosis, organelles lost most of their initial genome. Moreover, expression of the few remaining genes became tightly controlled by the nucleus through *trans*-acting protein factors that are required for post-transcriptional expression (maturation/stability or translation) of a single (or a few) specific organelle target mRNA(s). Here, we characterize the nucleus-encoded TDA1 factor, which is specifically required for translation of the chloroplast *atpA* transcript that encodes subunit α of ATP synthase in *Chlamydomonas reinhardtii*. The sequence of TDA1 contains eight copies of a degenerate 38-residue motif, that we named octotrico peptide repeat (OPR), which has been previously described in a few other *trans*-acting factors targeted to the *C. reinhardtii* chloroplast. Interestingly, a proportion of the untranslated *atpA* transcripts are sequestered into high-density, non-polysomic, ribonucleoprotein complexes. Our results suggest that TDA1 has a dual function: (i) trapping a subset of untranslated *atpA* transcripts into non-polysomic complexes, and (ii) translational activation of these transcripts. We discuss these results in light of our previous observation that only a proportion of *atpA* transcripts are translated at any given time in the chloroplast of *C. reinhardtii*.

Keywords: nucleo-chloroplast interactions, ATP synthase, translation regulation, ribonucleoprotein complexes, *Chlamydomonas reinhardtii*.

INTRODUCTION

After endosymbiosis, approximately 95% of the genes from the cyanobacterial progenitor of the chloroplast were either lost, when they became functionally irrelevant, or transferred to the nucleus of the host cell. In green eukaryotes, the chloroplast genome has only retained approximately 100 protein-encoding genes, whose products participate mostly in photosynthesis or expression of the chloroplast genome (Barkan and Goldschmidt-Clermont, 2000). Because of this extensive gene shuffling, photosynthetic protein complexes have a dual genetic origin, being composed of subunits encoded in either the organelle or nuclear genomes. They are assembled in stoichiometric amounts, despite the vast difference in gene dosage between the two compartments. Additionally, the *Chlamydomonas* nuclear genome is haploid, in contrast to approximately 80 copies of the chloroplast genome (Wollman *et al.*, 1999). Therefore, a stringent need for coordination of gene expression between these distinct genetic compartments has arisen during evolution.

Coordinated expression and stoichiometric accumulation of the various subunits of a photosynthetic complex are ensured by a variety of regulatory mechanisms. First, the subunits of a photosynthetic complex show concerted accumulation levels, as, in the absence of any of the major subunits, the other subunits are either rapidly degraded or poorly synthesized, because of an assembly-dependent regulation of translation known as Control by Epistasy of Synthesis (CES process) (reviewed by Choquet and Wollman, 2009). Furthermore, a plethora of nucleus-encoded proteins are specifically required for post-transcriptional expression of each chloroplast gene (Barkan and Goldschmidt-Clermont, 2000; Choquet and Wollman, 2002; Herrin and Nickelsen, 2004; Rochaix, 2004).

These nucleus-encoded factors have been classified into two major classes, depending on the post-transcriptional step that they primarily assist (Choquet and Wollman, 2002): M factors are involved in chloroplast mRNA maturation and stabilization, whereas T factors are required for mRNA translation. In *C. reinhardtii*, M factors such as MCA1, NAC2, MRL1 and MBB1 are required for stable accumulation of the *petA*, *psbD*, *rbcL* and *psbB* transcripts, respectively (Nickelsen *et al.*, 1994; Vaistij *et al.*, 2000b; Loiselay *et al.*, 2008; Johnson *et al.*, 2010), most often by protecting them from 5′ → 3′ exonucleolytic degradation. T factors are exemplified by TCA1 (Wostrikoff *et al.*, 2001; Raynaud *et al.*, 2007), TBC2 (Auchincloss *et al.*, 2002), RBP40 (Schwarz *et al.*, 2007) and TDA1 (this study), which activate translation of the chloroplast *petA*, *psbC*, *psbD* and *atpA* transcripts, respectively. However, M and T factors may have overlapping functions in transcript stability and translation activation, as documented for MCA1, which also acts as a translational enhancer for *petA* mRNA, while TCA1 participates in protection of the *petA* transcripts against exonucleolytic degradation (Loiselay *et al.*, 2008; Boulouis *et al.*, 2011).

We have previously shown that chloroplast mRNA availability is not limiting *per se* for organelle gene expression: after inhibition of chloroplast transcription by rifampicin for up to 6 h, trace amounts of *atpA* transcripts (5% or less compared with those found in untreated cells) were still able to sustain wild-type levels of synthesis of the *atpA* gene product, subunit α of chloroplast ATP synthase (Eberhard *et al.*, 2002). Conversely, untranslated *atpA* transcripts accumulate to wild-type levels in the nuclear *tda1*-F54 mutant, which is defective in *atpA* translation (Lemaire and Wollman, 1989; Drapier *et al.*, 1992). Together, these results suggest that the translational machinery uses only a fraction of the *atpA* transcripts accumulated in the chloroplast.

To obtain insights into the mechanisms that specifically control translation activation of only a subset of these *atpA* transcripts, we characterized TDA1, the activator of *atpA* mRNA translation. We observed that its C-terminal domain targets the *atpA* 5′ untranslated region (UTR) to activate translation. In addition, we provide evidence for TDA1-dependent trapping of a proportion of untranslated *atpA* transcripts into high-molecular-weight, non-polysomic, ribonucleoprotein complexes (RNPs) that relies on the N-terminal domain of TDA1. Differential interactions of these two domains of TDA1 with *atpA* mRNA thus trigger trapping of *atpA* transcripts within non-polysomic RNPs or activation for translation.

RESULTS

The molecular target of TDA1 lies in the 5′ UTR of the *atpA* transcript

As TDA1 is specifically required for synthesis of the chloroplast-encoded subunit α of ATP synthase (Lemaire and Wollman, 1989; Drapier *et al.*, 1992), we first determined its molecular target within the *atpA* transcript, using the chloroplast mutant strains *cA*α and *dAf* previously described by Choquet *et al.* (2003, 1998) and Drapier *et al.* (2007). In the *cA*α strain, the 5′*petA–atpA* chimera replaces the regular *atpA* gene, whereas the *dAf* strain expresses a 5′*atpA–petA* chimera instead of the endogenous *petA* gene (Figure 1a).

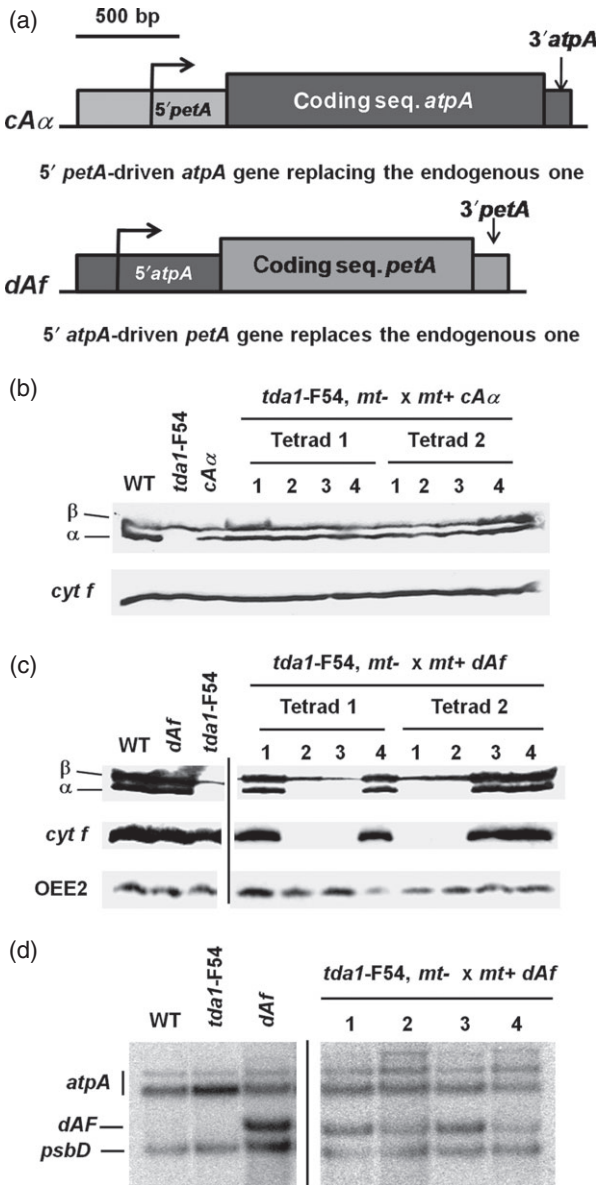

Figure 1. TDA1 targets the *atpA* 5′ UTR.
(a) Schematic representation of the *cA*α and *dAf* chimeras that replace the *atpA* and *petA* endogenous genes, respectively. Bent arrows indicate transcription start sites.
(b, c) Analysis of parents and offspring of the crosses between the strains *tda1*-F54 mt^- and *cA*α mt^+ (b) or *dAf* mt^+ (c). The wild-type strain is shown as a control. The accumulation of cytochrome *f* (b) and photosystem II subunit OEE2 (c) was used as loading controls.
(d) Transcript accumulation detected using probes specific for the *atpA* 5′ UTR and *psbD* coding sequence (loading control) in wild-type and parental strains and in tetrad 1 from (c).

These two strains (of mating-type mt^+) were crossed with the *tda1*-F54 mt^- nuclear mutant strain. Due to Mendelian inheritance of the *tda1*-F54 mutation, as opposed to uniparental inheritance of the chloroplast chimera from the mt^+ parent, half of the progeny from each cross expressed the *cA*α or *dAf* chimera in the *tda1*-F54 nuclear background. Nevertheless, all progeny from the *tda1*-F54 × *cA*α cross accumulated subunit α, whose expression is driven by the *petA* 5′ UTR (Figure 1b), showing that the *atpA* 5′ UTR is required for TDA1-dependent translation of the *atpA* gene. Analysis of the progeny from the cross *tda1*-F54 × *dAf*, which still carries the endogenous *atpA* gene, showed that half of them lacked expression of subunit α, as expected from the Mendelian segregation of the *tda1*-F54 mutation (members 2 and 3 and members 1 and 2 of tetrads 1 and 2, respectively) (Figure 1c). These same offspring also lacked cytochrome *f*, whose expression is driven by the 5′ UTR of *atpA* (Figure 1c), although the chimeric *petA* transcript accumulated in all progeny (Figure 1d). We therefore conclude that the 5′ UTR of *atpA* is the molecular target of the TDA1 factor, as it is required and sufficient to confer TDA1-dependent synthesis to a downstream coding region.

Cloning of the *TDA1* gene

To obtain further insights into the role(s) of TDA1, we cloned the *TDA1* gene. When plated on minimum medium under high light conditions, the *tda1*-F54 strain shows a high frequency of spontaneous reversion (>10^{-6}), preventing cloning of the gene by complementation. To bypass this flaw, we used the *mca1-1* mutant strain, which harbours a large deletion encompassing the *MCA1* gene (whose product is required for stable accumulation of *petA* mRNA) and an adjacent locus required for synthesis of subunit α (Figure 2a) (Loiselay *et al.*, 2008) that we suspected to be the *TDA1* locus.

First, we crossed the *tda1*-F54 mutant strain with the interfertile *Chlamydomonas grossii* species (also known as S1D2 or CC-2290), which shows suitable profusion of genomic polymorphism for linkage analysis (Rymarquis *et al.*, 2005), including one sequence-tagged site (STS) within the *MCA1* gene. In each of the 41 tetrads analysed, one clone that was identified as having inherited the *tda1*-F54 mutation because it showed defective photoautotrophic growth, was subjected to PCR amplification using appropriate primers (Rymarquis *et al.*, 2005; see also the primers used in this study as listed in Table S1). Of these, 37 inherited the STS typical of the *C. reinhardtii MCA1* gene, indicating tight genetic linkage between the *tda1*-F54 mutation and the *MCA1* locus. Hence, the large deletion in strain *mca1-1* that removes at least 10 kbp downstream of the *MCA1* gene probably extends into *TDA1* as well.

In a second step, we co-transformed the *mca1-1* deletion double mutant with the *MCA1* gene (Loiselay *et al.*, 2008) and the *ble* selectable marker that confers resistance to the antibiotic Zeocin (Invitrogen, http://www.invitrogen.com/) (Stevens *et al.*, 1996). Transformed clones selected for Zeocin resistance were further screened by fluorescence

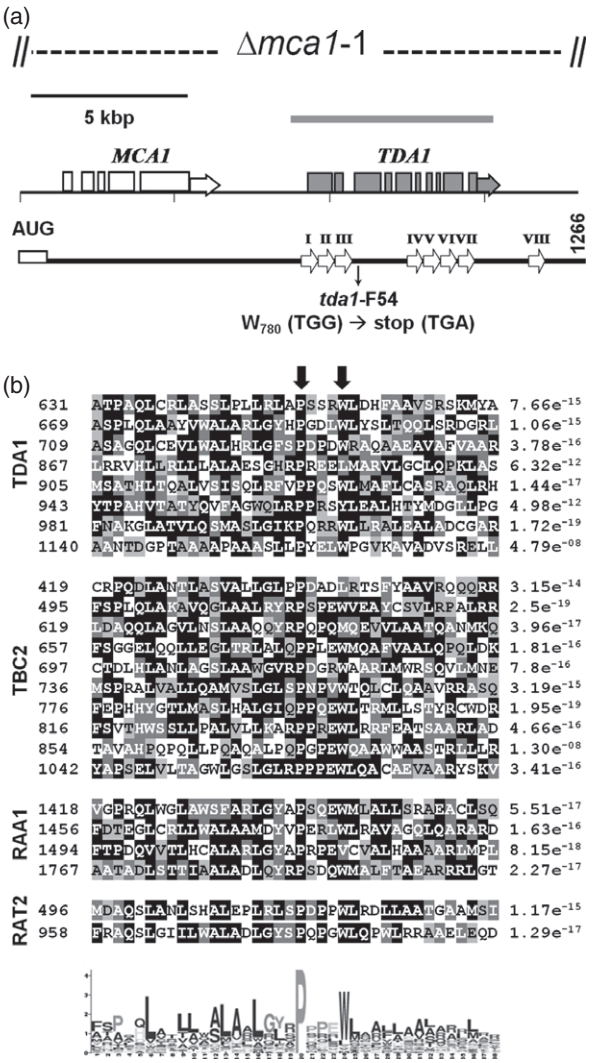

Figure 2. The *TDA1* gene encodes a 1266 residue protein containing eight OPR motifs in its C-terminal part.
(a) Structure of the *MCA1/TDA1* genomic region. Exons are shown as grey boxes for *TDA1* and white boxes for *MCA1*. Arrows indicate the *MCA1* or *TDA13′* UTRs. The deletion in strain *mca1-1* is indicated by a dashed line. Both borders of the deletion lie outside the figure. The thick grey bar indicates the location of the genomic fragment used to restore phototrophy in strain *tda1*-Δ. A diagram of the TDA1 protein is shown below, indicating the position of the W780 → stop codon mutation found in strain *tda1*-F54, as well as the position of the OPR repeats (thick white arrows). The putative transit peptide is indicated by a white rectangle.
(b) Alignment of the OPR motifs identified by the MEME program in TDA1, TBC2, RAA1 and RAT2 visualized with the GeneDoc software (http://www.nrbsc.org/gfx/genedoc/) using default parameters. Residues with the highest, second highest and third highest counts in each column are identified in black, dark grey and light grey, respectively. Black arrows indicate the most conserved residues (the first P and the W) in the consensus PPPEW motif found in OPRs. *P* values describe the statistical significance of each OPR occurrence. The consensus sequence of the OPR motif derived by the MEME software is indicated below the alignments.

for recovery of cytochrome b_6f activity. These clones, hereafter referred to as *tda1*-Δ, accumulated cytochrome *f*, indicating successful complementation of the *mca1* mutation (Figure 3b). However, they still lacked subunit α expression, and were used, in a third step, as recipient strains in transformation experiments aimed at complementing the ATP synthase defect. As shown in Figure 3(b) for three independent transformants selected for phototrophic growth, complementation by plasmid *pTDA1* containing the putative *TDA1* gene restored subunit α expression. To confirm that we had cloned the *TDA1* gene, we sequenced this locus in strain *tda1*-F54 and found a single base pair change that substituted codon W780 by a stop codon, causing premature translation termination (Figure 2a). This single nonsense mutation may explain the high reversion rate of strain *tda1*-F54 when plated on minimum medium.

TDA1 contains OPR repeats in its C-terminus that are necessary and sufficient to activate *atpA* mRNA translation

Sequencing of cDNAs derived from the locus (see Experimental procedures) showed that the *TDA1* gene comprises ten exons and encodes a 1266 residue protein (Figure 2a and Figure S1), that was predicted to be targeted to the chloroplast using the programs Predotar (Small *et al.*, 2004), TargetP (Emanuelsson and von Heijne, 2001), WolfPsort (Horton *et al.*, 2007) and MultiLoc (Hoglund *et al.*, 2006). An in-frame stop codon, 24 nucleotides upstream of the initiation codon, confirmed that we had cloned the complete coding sequence. The secondary structure prediction software Psipred version 2.6 (http://bioinf.cs.ucl.ac.uk/psipred/) suggested that the TDA1 protein potentially comprises two different domains.

In addition to the putative chloroplast-targeting sequence of 67 residues, the N-terminal domain (up to residue 594) has a coiled-coil structure with no other obvious structural features or motifs. By contrast, the C-terminal domain contains paired α helices (Figure S2). Some of these paired α helices overlap with eight repeats of a degenerate 38-residue motif that was identified using the MEME software (http://meme.sdsc.edu/meme4_5_0/intro.html) and that we named OPR, for octotrico peptide repeat (Figure 2b and Figures S1–S4). OPRs show a typical degenerate consensus of five residues, PPPEW, previously identified in *C. reinhardtii* in the sequence of the nucleus-encoded factors TBC2, RAA1 and RAT2, which are required for translation of *psbC* (Auchincloss *et al.,* 2002), trans-splicing of *psaA* (Merendino *et al.,* 2006) and maturation of the *tscA* transcripts (Balczun *et al.,* 2005), respectively. The two most conserved residues, the first P and the W, are indicated by black arrows in Figure 2(b) and Figure S3 and boxed in Figure S2.

We wished to determine whether these structurally contrasting N- and C-terminal domains of TDA1 play distinct functional roles, and therefore generated two plasmids,

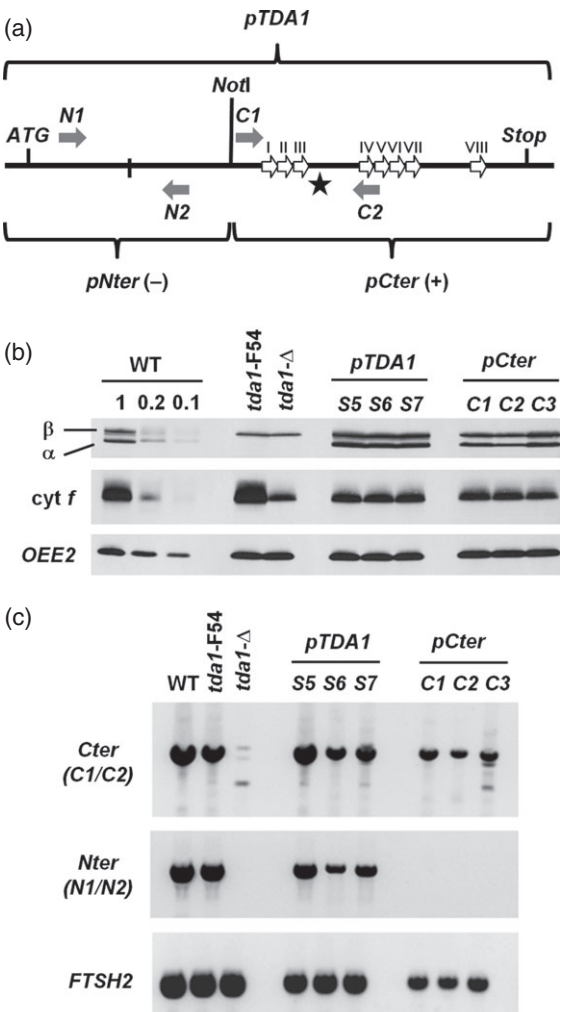

Figure 3. The OPR-containing C-terminal part of TDA1 is sufficient to activate translation of the *atpA* transcript.
(a) Constructs *pTDA1*, *pNter* and *pCter*, used to complement strain *tda1*-Δ, (see text). Phototrophic transformants were recovered when plasmids *pTDA1* and *pCter* (+) were used, but not when plasmid *pNter* (–) was used. Grey arrows indicate the position of the primers used for PCR analysis of the complemented strains. The star indicates the position of the W780 → stop mutation in the *tda1*-F54 strain.
(b) Immunoblot analysis of transformants. Three independent transformants are shown for each transformation (*pTDA1*: *S5, S6* and *S7*; *pCter*: *C1, C2 and C3*). Accumulation of ATP synthase subunits α and β, cytochrome *f* and OEE2 (loading control) was assessed using specific antibodies. The reduced accumulation of cytochrome *f* in strain *tda1*-Δ and derivatives is due to ectopic complementation of the *MCA1* deletion.
(c) PCR analysis of the same transformants. A PCR product was found in both types of transformants using primers C1/C2, hybridizing to the sequence coding for the TDA1 C-terminus. When using primers N1/N2 complementary to the upstream part of *TDA1*, amplification was only observed in transformants obtained using *pTDA1*, but not for those obtained using *pCter*. Analysis of strain *tda1*-F54, carrying a point mutation, shows both bands, whereas the deletion mutant *tda1*-Δ shows neither. *FTSH2* was used as a positive PCR control.

pNter and *pCter*, respectively, that contain *TDA1* sequences upstream and downstream of a unique *Not*I site that is located upstream of the region encoding the OPR repeats (Figure 3a). Plasmids *pTDA1* and *pCter*, but not *pNter*,

complemented the *tda1*-Δ strain (Figure 3b). The absence of the 5′ part of the *TDA1* gene in the phototrophic transformants recovered after transformation with *pCter* was confirmed by PCR (Figure 3c). Thus, the last 717 residues of TDA1, comprising the OPR motifs, are sufficient to activate *atpA* mRNA translation. Conversely, the absence of the five last OPRs in the truncated TDA1 protein expressed in strain *tda1*-F54 (Figure 3a) prevents synthesis of subunit α.

atpA transcripts are found in non-polysomic RNPs in the *tda1*-F54 strain

Defective synthesis of subunit α, as monitored by short ^{14}C-acetate pulse-labelling experiments in strains *tda1*-F54 (Lemaire and Wollman, 1989; Drapier *et al.*, 1992) or *tda1*-Δ (data not shown), together with localization of the molecular target of TDA1 in the *atpA* 5′ UTR, suggested that TDA1 is required for translation initiation. To further substantiate this hypothesis, we compared the loading of *atpA* transcripts onto polysomes in wild-type and *tda1* mutant strains, after sedimentation of solubilized cellular extracts on sucrose gradients, as described previously (Minai *et al.*, 2006). Use of chloramphenicol (CAP), which inhibits the peptidyl transferase activity of prokaryotic ribosomes and stabilizes polysomes (Weber and DeMoss, 1966; Xaplanteri *et al.*, 2003), limits polysome run-off during experiments. In *C. reinhardtii*, CAP has been used prior to polysome purification in pre-treatments ranging in duration from 10 min (Hoober and Blobel, 1969) or 20 min (Bolli *et al.*, 1981) to 1 h (Chua *et al.*, 1973). After 10 min pre-treatment with CAP, polysomes are found in 'heavy' fractions (fractions 1–5: sucrose concentration >30%), while monosomes and EDTA-dissociated 30S and 50S subunits are found in fractions 4–6 and 6–10, respectively (see Figure S5 for a characterization of sedimentation profiles).

As shown in Figure 4, *atpA* and *rbcL* transcripts from the wild-type were distributed approximately equally between 'heavy' fractions and 'light' fractions (fractions 6–10: sucrose concentration <30%), as was the *rbcL* transcript from strains *tda1*-F54 and *tda1*-Δ. In strain *tda1*-Δ, as expected from its defect in *atpA* mRNA translation, *atpA* transcripts were hardly detectable in fractions 1–5. Most unexpectedly, although *atpA* transcripts strains were significantly shifted towards lighter fractions when compared to the wild-type, 13% remained in 'heavy' fractions in the *tda1*-F54 strain, which does not synthesize subunit α either (Figure 4b).

These contrasting distributions of *atpA* transcripts between the *tda1*-F54 and *tda1*-Δ strains, both of which are defective for *atpA* mRNA translation, suggested that the truncated protein expressed in strain *tda1*-F54 accumulates to some extent and retains its ability to interact, directly or indirectly, with *atpA* transcripts.

Because we suspected that inhibition of chloroplast translation *in vivo* could have pleiotropic effects on chloroplast transcript metabolism, we shortened the exposure of the cells to CAP by adding this antibiotic to the resuspension buffer only, as described by Rott *et al.* (1998). In the wild-type, reduced exposure to CAP (Figure 5a,b) significantly decreased the proportion of RNAs found in heavy fractions (compare Figure 5a,b with Figure 4a,b): <10% of *rrnS* rRNA and only 27% of the *atpA* transcripts were found in fractions 1–5, compared to 45% and 58%, respectively, after a 10 min pre-treatment. These differences can be accounted for in two ways: either short exposure to CAP lowers the yield of polysomes, or RNA metabolism is altered by longer CAP pre-treatment, questioning the physiological significance of the distributions shown in Figure 4.

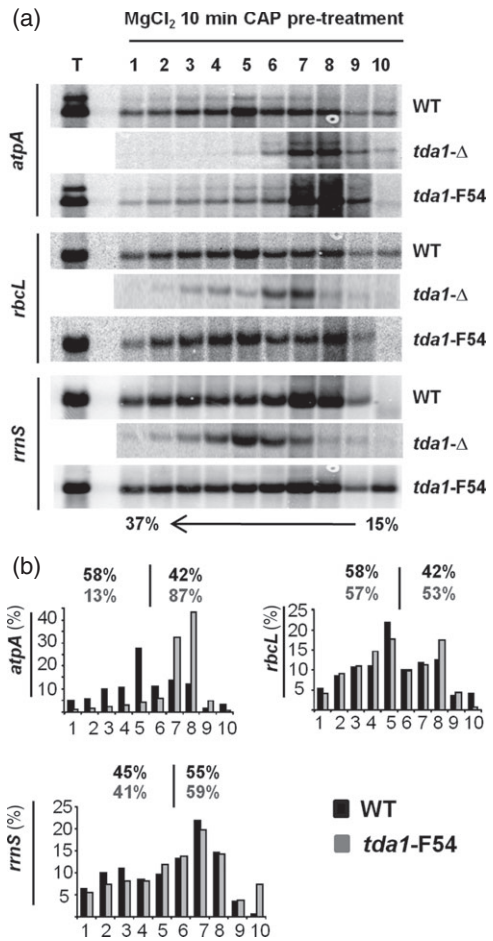

Figure 4. *atpA* transcripts are found in heavy fractions in the *tda1*-F54 strain.
(a) Solubilized whole-cell extracts (T) from wild-type, *tda1*-Δ and *tda1*-F54 strains pre-treated for 10 min with CAP (100 μg ml^{-1}) were loaded on sucrose gradients. After ultracentrifugation, ten fractions were collected and the transcripts present in each were analysed by RNA blotting using the gene-specific probes indicated on the left. In this experiment, the gradients were slightly shorter (i.e. 15–37% sucrose concentration) than in the other experiments performed in this study (i.e. 15–45% sucrose concentration).
(b) Distribution of *atpA*, *rbcL* and *rrnS* transcripts in wild-type and *tda1*-F54 strains, quantified from ^{33}P labelling, and expressed as the percentage of total transcript found in each fraction.

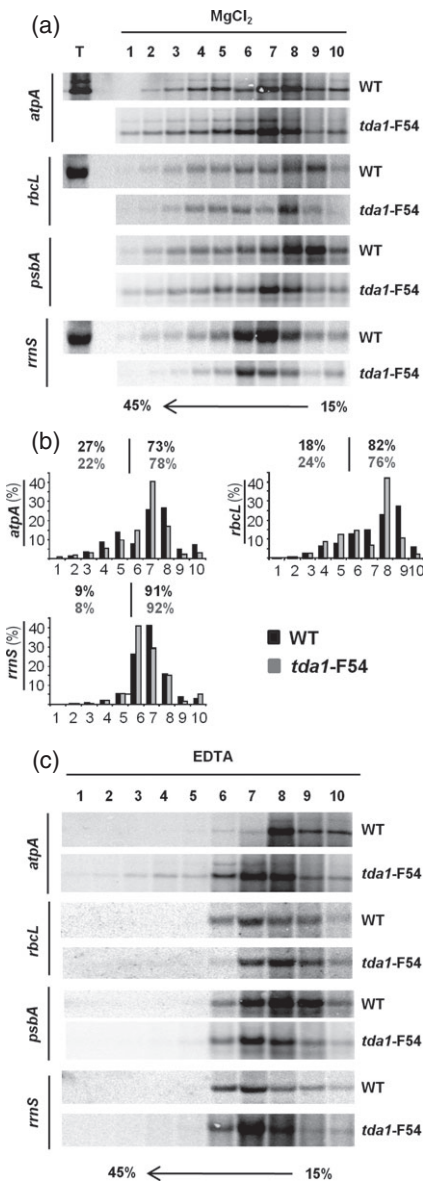

Figure 5. In the absence of CAP pre-treatment, *atpA* transcripts are still found in heavy fractions that are partly EDTA-resistant, in strain *tda1*-F54.
(a) Comparative distribution of *atpA*, *rbcL*, *psbA* and *rrnS* transcripts in wild-type and *tda1*-F54 strains assessed as in Figure 4, except that CAP was added in the resuspension buffer only. T represents the total RNA extract of a wild-type strain.
(b) Distribution of *atpA*, *rbcL* and *rrnS* RNAs in these strains, expressed as in Figure 4.
(c) Distribution of *atpA*, *rbcL* and *rrnS* transcripts in wild-type and *tda1*-F54 extracts incubated in the presence of EDTA (20 mM final concentration), analysed as in Figure 4.

Using these new experimental conditions, we re-examined the distribution of *atpA* transcripts in the *tda1*-F54 strain (Figure 5a), and detected 22% of the *atpA* transcripts in fractions 1–5 (Figure 5b), a proportion even higher than under the previous conditions (Figure 4b). Hence, in the absence of pre-incubation with CAP, the content in *atpA* transcripts did not decrease in 'heavy' fractions from strain *tda1*-F54, in contrast to *rbcL* and *rrnS* RNAs, and in marked contrast to the behaviour of these three RNAs in the wild-type (compare Figure 5 with Figure 4). We also note that the distribution of *atpA* transcripts was bimodal in the wild-type, with a first peak in fraction 5, probably the peak of polysomes, and a second peak in fraction 8, most likely corresponding to mRNA that is not associated with ribosomes (Figure 5a,b). This bimodal distribution was not observed in strain *tda1*-F54.

After EDTA treatment of the wild-type extracts, *atpA*, *rbcL*, *psbA* and *rrnS* transcripts were no longer found in 'heavy' fractions 1–5 but were found exclusively in 'light' fractions 6–10 (Figure 5c). In marked contrast, EDTA-treated *tda1*-F54 extracts reproducibly contained detectable *atpA* transcripts in fractions 1–5 (Figure 5c). These EDTA-insensitive heavy fractions were specific for *atpA*, as neither *rbcL* nor *psbA* transcripts were found in fractions 1–5 after EDTA treatment. Most importantly, *rrnS* RNA was not detected in fractions 1–5 after EDTA treatment of the *tda1*-F54 extracts, confirming the absence of ribosomes in these fractions.

The presence of untranslated *atpA* transcripts in heavy fractions from strain *tda1*-F54 but not from strain *tda1*-Δ (Figure 4 and data not shown) suggests the existence of non-polysomic RNPs that are partially resistant to EDTA, formation of which critically depends on the N-terminal domain of TDA1, which is expressed in the former strain only.

An untranslatable $atpA_{St}$ transcript is also associated with non-polysomic RNPs

To obtain independent evidence for the presence of non-polysomic RNPs containing untranslated *atpA* transcripts, we constructed an inherently untranslatable version of the *atpA* gene in which the *atpA* initiation codon was substituted by a stop codon (Figure 6a). $atpA_{St}$ transformants, recovered after transformation of the wild-type strain with this mutated *atpA* gene (associated with an *aadA* spectinomycin resistance cassette to allow selection of transformed cells), accumulated the mutated transcript to levels comparable to those of the *atpA* transcript in the wild-type (Figure 6b). As expected from substitution of the *atpA* initiation codon by a stop codon, this mutant strain showed neither accumulation nor synthesis of subunit α in 5 min ^{14}C-acetate pulse-labelling experiments (Figure 6c).

We analysed the distribution of $atpA_{St}$ transcripts along sucrose gradients in the absence of CAP pre-treatment. Approximately 18% of the untranslatable $atpA_{St}$ transcripts were readily detected in heavy fractions 1–5 under $MgCl_2$ conditions (Figure 6d), a proportion similar to that observed in wild-type and *tda1*-F54 strains. The bimodal distribution of *atpA* transcripts observed in the wild-type was lost in strain $atpA_{St}$. Thus, under $MgCl_2$ conditions, the $atpA_{St}$

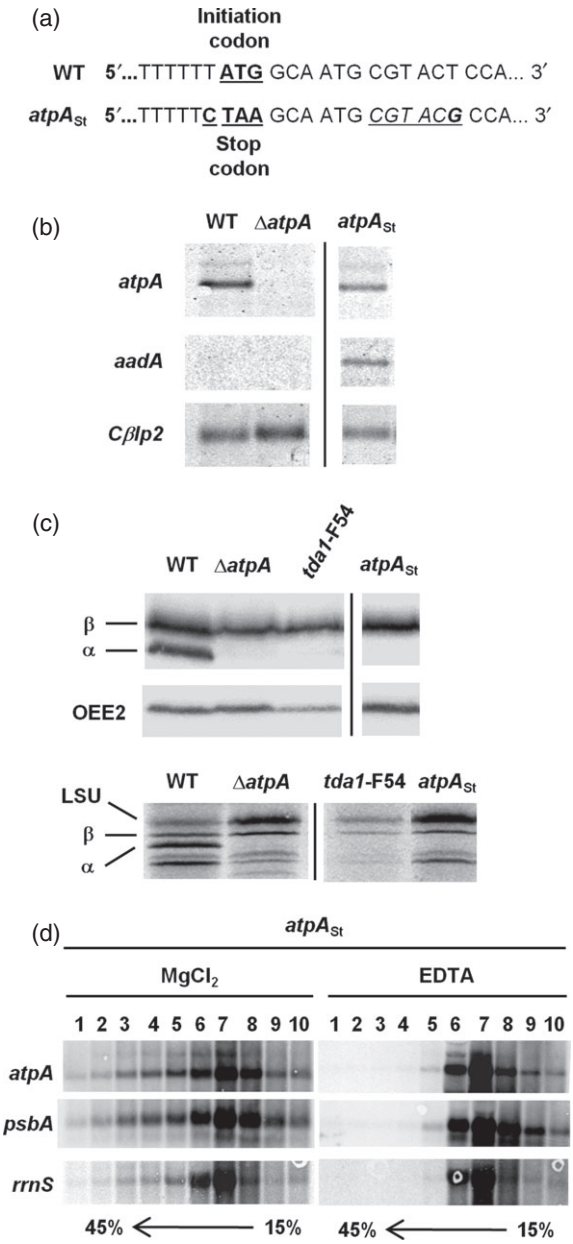

Figure 6. Expression of the untranslatable *atpA*$_{St}$ transcript.
(a) Changes introduced into the *atpA* gene: mutated nucleotides are shown in bold. The initiation codon (underlined) was mutated to a stop codon, and the T at position-1 (possibly involved in translational activation; Esposito *et al.*, 2003) was mutated to C. A silent mutation was also introduced to create a *Bsi*WI RFLP marker (underlined).
(b) The *atpA*$_{St}$ transcript accumulates to levels comparable to those of the *atpA* transcript in the wild-type, but is absent in the control deletion strain Δ*atpA*. *aadA* is expressed from the spectinomycin resistance cassette present in the *atpA*$_{St}$ strain only, Cβlp2 provides a loading control.
(c) Accumulation (top) and rate of the synthesis assessed by 5 min ^{14}C-acetate pulse-labelling experiments (bottom) for the ATP synthase subunits α and β in the mutant strains Δ*atpA*, *tda1*-F54 and *atpA*$_{St}$ and the wild-type. Accumulation of OEE2 provides a loading control (top). The position of the newly synthesized LSU is indicated (bottom).
(d) Distribution of *atpA*$_{St}$, *psbA* and *rrnS* transcripts in strain *atpA*$_{St}$, assessed as in Figure 5 under MgCl$_2$ and EDTA conditions.

transcript behaves in a wild-type nuclear genetic context, as did the *atpA* transcript in the nuclear mutant *tda1*-F54 (compare with Figure 5).

After EDTA treatment, the *atpA*$_{St}$ transcript, although mostly found in fractions 6 and 7, was still detected in fractions 3–5 (Figure 6d), at variance with the wild-type *atpA* transcript, which was exclusively found in fractions 6–10 (Figure 5c). Thus, these *atpA*$_{St}$ transcript-containing heavy fractions also showed lower sensitivity to EDTA, but this was less pronounced than in strain *tda1*-F54. Together, these observations suggest that *atpA*$_{St}$ transcripts are part of non-polysomic RNPs that are slightly more resistant to EDTA than polysomes are.

Trapping of untranslated *atpA* transcripts into non-polysomic RNPs depends on the *atpA* 5′ UTR.

The above observations suggest that some untranslated *atpA* transcripts may associate with non-polysomic RNPs either (i) in the absence of the C-terminal OPR-containing region of TDA1 required for translation activation (strain *tda1*-F54), or (ii) when inherently untranslatable (strain *atpA*$_{St}$). As the *atpA* 5′ UTR is the target for TDA1-mediated translation activation (Figure 1), this region may also be involved in formation of non-polysomic RNPs containing untranslated *atpA* transcripts. We therefore used the double mutant (*tda1*-F54 *dAf*) recovered from the cross *tda1*-F54 × *dAf* (Figure 1) to compare the distribution of the endogenous *atpA* and chimeric 5′ *atpA–petA* transcripts in the nuclear *tda1*-F54 context, in which none of them can be translated. As previously observed in strain *tda1*-F54 (Figures 4 and 5), *atpA* transcripts from the *tda1*-F54 *dAf* mutant were found in heavy fractions 1–5 under both MgCl$_2$ and EDTA conditions (Figure 7). Most interestingly, the chimeric 5′ *atpA–petA* transcript, which only retains the *atpA* 5′ UTR,

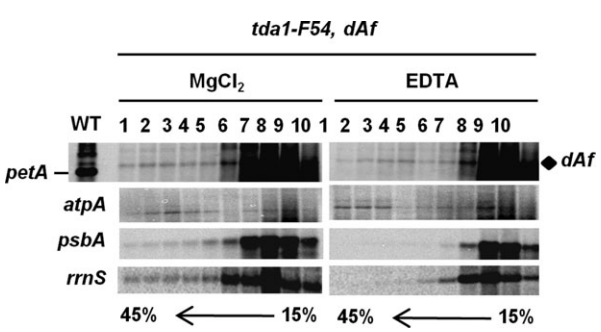

Figure 7. The *atpA* 5′ UTR is sufficient to recruit reporter coding regions to non-polysomic RNPs. Distribution of *dAf*, *atpA*, *psbA* and *rrnS* transcripts under MgCl$_2$ and EDTA conditions in the *tda1*-F54 *dAf* strain that expresses the 5′ *atpA–petA* chimera in the nuclear *tda1*-F54 background. In the first lane, a wild-type extract (WT) is shown for ease of comparison. In strain *tda1*-F54 *dAf*, the regular *petA* transcript is absent and is replaced by the slightly larger 5′ *atpA–petA* chimeric transcript (indicated by a diamond). The *psbA* transcript provides an EDTA-sensitive control.

was also found in fractions 1–5 under $MgCl_2$ and EDTA conditions. Thus, the *atpA* 5′ UTR is not only involved in the TDA1-dependent translation activation of 5′ *atpA*-driven transcripts (Figure 1), but also in their recruitment into non-polysomic RNPs when untranslated.

DISCUSSION

Only a subset of the *atpA* transcripts is translated at a given time

After evolution from a cyanobacterial genome, which is mainly regulated at the transcriptional level, to a chloroplast genome, whose expression is mostly regulated post-transcriptionally, some chloroplast transcripts may be produced at levels above those required to sustain wild-type levels of protein synthesis. The chloroplast *atpA* transcript in *C. reinhardtii* is a particularly striking example, as its accumulation may be decreased more than 10-fold with no effect on its rate of translation (Eberhard *et al.*, 2002). The accumulation of other chloroplast transcripts such as *petA*, *petD*, *psaA* or *atpB* mRNAs was also strongly reduced by rifampicin treatment, with very limited effect on the rate of synthesis of their gene products (Eberhard *et al.*, 2002). However, this behaviour should not be taken as a general rule for chloroplast gene expression, as reduced accumulation of *psbD* mRNA because of a mutation in its promoter region was accompanied by a similar reduction in the rate of synthesis of the encoded protein D2 (Klinkert *et al.*, 2005), suggesting that mRNA accumulation is limiting for translation of this gene. Our characterization of the nucleus-encoded *trans*-acting factor TDA1, which is specifically required to activate translation of *atpA* transcripts through specific interaction with the *atpA* 5′ UTR, provided the opportunity to better understand how a limited fraction of *atpA* transcripts may be used to drive normal rates of translation, while the rest are trapped as untranslated transcripts.

OPR-containing proteins may constitute a large family of nucleus-encoded regulators of organelle gene expression in *Chlamydomonas*

Molecular characterization of TDA1 indicated that it is a bi-functional protein that harbours a coiled-coil N-terminal region and a C-terminal that contains eight degenerate repeats of 38 residues, called OPR (octotrico peptide repeats). A true orthologue, with high amino-acid identity throughout the sequence, was only found in the closely related alga *Volvox carterii* (Figure S3).

Although the N-terminal part of TDA1 does not show significant homology to any proteins in *Chlamydomonas* or other organisms (other than its orthologue in *Volvox*), OPR domains are found in nucleus-encoded factors that have been described previously as governing translation (TBC2; Auchincloss *et al.*, 2002), maturation (RAT2; Balczun *et al.*, 2005) and *trans*-splicing (RAA1; Merendino *et al.*, 2006) of chloroplast transcripts in *C. reinhardtii*. Systematic searches of the nuclear genome of *C. reinhardtii* using the MEME/MAST software package (http://meme.sdsc.edu/meme4_5_0/intro.html) identified more than 40 OPR proteins in this organism (see Table S2), most of which (32 of 42) were predicted as organelle-targeted by the program WolfPsort (Horton *et al.*, 2007). Sequence conservation between these various OPR proteins was essentially restricted to the OPR repeats. We only found few OPR proteins in the Arabidopsis genome (data not shown).

Although it remains to be tested experimentally, secondary structure predictions performed on TDA1 (Figure S2) and other OPR proteins (data not shown) suggest that the OPR motifs consist of arrayed α helices. OPRs are thus new members of the 'α-solenoid' superfamily of proteins, which contain superhelical structures formed of tandem antiparallel α-helical units. This superfamily, which comprises ankyrin repeat proteins and Puf domain RNA-binding proteins, also includes pentatrico peptide repeat and tetratrico peptide repeat proteins, whose function in post-transcriptional expression of organelle mRNAs (including editing, splicing, maturation, protection against exonucleotidic degradation and translation) is well documented (reviewed by Das *et al.*, 1998; Blatch and Lassle, 1999; Schmitz-Linneweber and Small, 2008). Pentatrico and tetratrico peptide repeats presumably participate in RNA/protein and protein/protein interactions, respectively, although organellar tetratrico peptide repeats have been proposed to mediate RNA/protein interactions (Sane *et al.*, 2005). OPR repeats may also interact specifically with organelle transcripts, as suggested by the function of the TBC2, RAT2 and RAA1 factors. Accordingly, the C-terminal part of TDA1, containing the OPR repeats, is itself sufficient to promote translation of *atpA* transcripts.

The N-terminal part of TDA1 recruits untranslated *atpA* transcripts into non-polysomic RNPs, transiently formed prior to translational activation

In contrast to its OPR-containing C-terminus, the N-terminal part of TDA1 is dispensable for translational activation but appears to be required to recruit untranslated *atpA* transcripts into non-polysomic RNPs, as suggested by the distribution of *atpA* transcripts in heavy RNP complexes in mutant strains defective for *atpA* translation. Although *atpA* transcripts were not found in RNPs in the complete absence of TDA1 (strain *tda1*-Δ), a proportion of these were associated with RNPs in translation-defective strains that still express TDA1 ($atpA_{St}$) or its N-terminal domain only (*tda1*-F54 and *tda1*-F54 *dAf*) (Figure 8). These non-polysomic RNPs were more resistant to EDTA than polysomes are, although to a lower extent in strain $atpA_{St}$ than in strains *tda1*-F54 and *tda1*-F54 *dAf* (compare Figure 5d and Figure 6d). Possibly, the RNPs formed in the presence of full-

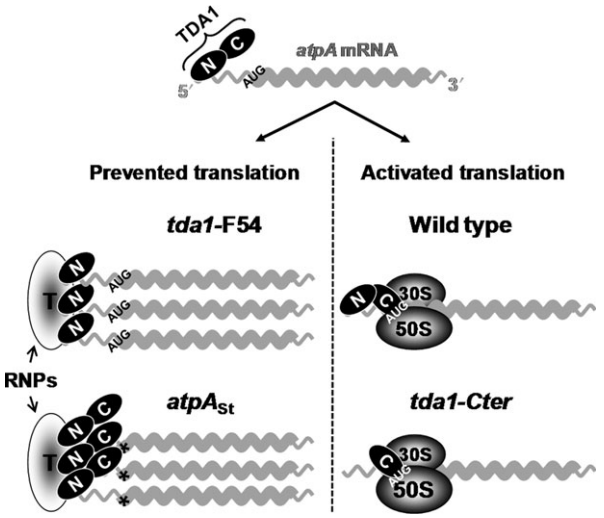

Figure 8. Working model for TDA1-dependent trapping and translation activation of *atpA* transcripts.
In the wild-type, the N-terminal region of TDA1 targets the 5′ UTR of the *atpA* transcripts, which are then activated for translation through interaction with the OPR-containing C-terminal part of TDA1. This C-terminal region alone is sufficient to promote translation (strain *tda1-Cter*). When *atpA* transcripts cannot be translated (in the absence of the TDA1 C-terminal region; strain *tda1*-F54) or when the AUG initiation codon is missing (indicated by an asterisk in strain *atpA*$_{St}$), a proportion of them are trapped into non-polysomic RNPs, formation of which critically depends on the TDA1 N-terminal region and may also involve other, so far uncharacterized, proteins, indicated by 'T' for trapping.

length TDA1 (strain *atpA*$_{St}$) or its truncated N-terminal domain only (strains *tda1*-F54 or *tda1*-F54, *dAf*) do not have exactly the same composition, explaining their different sensitivity to EDTA. Alternatively, the mutation of the *atpA* initiation codon may have weakened the interaction between the *atpA* 5′ UTR and TDA1 when compared to the other strains that retained a wild-type *atpA* 5′ UTR. In this case, formation of non-polysomic RNPs containing untranslated *atpA* transcripts would depend on the N-terminal part of TDA1 and an unaltered target of TDA1 in the *atpA* 5′ UTR (Figure 8). The interaction between TDA1 and the *atpA* 5′ UTR would thus involve two successive steps at the translation initiation site: (i) pre-recruitment of untranslated *atpA* transcripts into non-polysomic RNPs by the N-terminal domain of TDA1, followed by (ii) translation activation upon transfer to polysomes by the C-terminal domain of TDA1. In the absence of TDA1 (strain *tda1*-Δ), neither step would occur. In the wild-type, on-going translation would prevent accumulation of these transient RNPs, whose level remains below the detection threshold in EDTA-treated samples. By contrast, non-polysomic RNPs would be stabilized when translation activation is impaired, as in the absence of the TDA1 C-terminal OPR-containing region (strain *tda1*-F54) or when mutating the translation initiation codon (strain *atpA*$_{St}$).

Non-polysomic RNPs may regulate translation rates in the chloroplast by fine-tuning the amount of transcripts directed towards translation

In *Chlamydomonas*, high-molecular-weight, non-polysomic complexes containing chloroplast RNAs and nucleus-encoded factors have been described previously (Vaistij *et al.*, 2000a; Auchincloss *et al.*, 2002; Dauvillee *et al.*, 2003), such as those containing *psbD* mRNA associated with the nucleus-encoded factors NAC2 and RBP40 (Schwarz *et al.*, 2007) or *petA* transcripts together with TCA1 and MCA1 (Boulouis *et al.*, 2011). However, these non-polysomic RNPs are much smaller (<1000 kDa) than the *atpA*-containing RNPs described here and are sensitive to EDTA. Although they appear to be critical for translation activation, their function in storage/recruitment of untranslated mRNAs has not yet been documented.

Storage of untranslated cytosolic transcripts, sometimes as a form of translation pre-activation, is well documented: in many eukaryotic cells (including plants and mammals) subjected to stress conditions, mRNAs localize to stress granules that sequester translationaly repressed transcripts (Anderson and Kedersha, 2008, 2009a,b). In mammals, stress granules constitute a dynamic compartment involved in rapid routing of transcripts from and to polysomes, and to RNPs and processing bodies for storage or degradation (Kedersha *et al.*, 2000, 2005). Interestingly, stress granule-like structures have recently been identified in mitochondria (Sasarman *et al.*, 2010) and in the chloroplast of *C. reinhardtii*, where they were named cpSGs (Uniacke and Zerges, 2008). cpSGs participate in the trafficking of chloroplast transcripts from and to polysomes and contain untranslated mRNAs, such as those that accumulate upon lincomycin treatment and polysome dissociation (Uniacke and Zerges, 2008). cpSGs, or similar structures, may sequester untranslated *atpA* transcripts and direct them to polysomes when needed. Although we did not observe *atpA*-containing high-density RNPs in lincomycin-treated wild-type cells under our experimental conditions (data not shown), we note that only 10% of the tested cells showed formation of cpSGs upon lincomycin treatment in the study by Uniacke and Zerges (2008), which may be below the detection level for RNPs in sucrose gradients.

As shown in Figure S4, OPR repeats are probably related to the RNA-binding domain abundant in Apicomplexans (RAP) domain (Lee and Hong, 2004). In addition, TDA1, as several other OPR proteins, contains a region related to the FAST1 kinase-like domain (Tian *et al.*, 1995) (see Table S2). The presence of the latter domain in TDA1 raises the possibility that its function may involve reversible phosphorylation processes that have not previously been considered with regard to the action of *trans*-acting factors for organelle gene expression. Most interestingly, FAST/RAP-containing proteins have been proposed to play a prominent role in formation of cytosolic stress granules, in which

mRNAs are sequestered upon stalled initiation of translation (Anderson and Kedersha, 2002a,b). Moreover, at least five FAST kinase-like domain-containing proteins are targeted to mitochondria, and one of them, FASTKD3, interacts with the RNA processing and translation machineries (Simarro *et al.*, 2010). Thus, some OPR proteins, apparently related to the RAP/FAST proteins involved in processing and translation of cytosolic and mitochondrial transcripts, may play a similar role in the chloroplast, and may be major actors in chloroplast RNA metabolism.

Storage of untranslated transcripts may allow a rapid supply of translatable transcripts when cell physiology requires increased production of a photosynthetic protein. However, variations in the cellular demand for ATP synthase have not been reported to date in *C. reinhardtii*. Instead, pre-recruitment and trapping of *atpA* transcripts could enable the organism to cope with a fluctuating requirement for subunit α synthesis during ATP synthase assembly. Over the course of CF1 biogenesis in *Chlamydomonas*, synthesis of subunit α is activated *in trans* by subunit β (Drapier *et al.*, 2007). The fate of the *atpA* transcripts, before activation for translation by subunit β, has not yet been investigated. The bi-functional properties of TDA1 may help to better understand the status of these *atpA* transcripts: TDA1 could be a major regulatory factor in biogenesis of the chloroplast ATP synthase in *C. reinhardtii*, being responsible for recruitment of newly transcribed *atpA* transcripts into non-polysomic RNPs until the availability of 'free' subunit β allows TDA1 to activate subunit α translation. In this model, interactions between TDA1 and subunit β are critical for the switch of TDA1 from storage mode to translational activation mode. Further biochemical experiments are underway to investigate the interplay between subunit β and TDA1 in *atpA* mRNA translation activation.

EXPERIMENTAL PROCEDURES

Strains and growth conditions

Wild-type, derived from 137c, mutant and transformed strains of *C. reinhardtii* were grown in Tris/acetate phosphate (TAP) medium, pH 7.2 (Harris, 1989) under continuous low light (10 μE m^{-2} sec^{-1}). Crosses were performed as described by Harris (1989). The nuclear mutant strains *tda1*-F54 (Lemaire and Wollman, 1989; Drapier *et al.*, 1992) and *mca1-1* (Loiselay *et al.*, 2008) were used in this study, as well as the chloroplast transformants *dAf* [complex d (ATP synthase) 5′ *atpA*-driven cytochrome *f* (Choquet *et al.*, 1998; Drapier *et al.*, 2007)], *cA*α [cytochrome b_6f 5′ *petA*-driven subunit α, formerly called FAAA by Choquet *et al.* (2003)] and Δ*atpA* (Drapier *et al.*, 2002).

DNA constructs

Construction of the $atpA_{St}$ *plasmid.* Plasmid *pADE*, containing a 486 bp *Dra*II/*Eco*RI fragment of the *C. reinhardtii* chloroplast genome encompassing the *atpA* initiation codon sub-cloned into vector pBSKS-digested with *Sma*I and *Eco*RI, was used as template for PCR mutagenesis with primers AS1 and AS2 (see Table S1). The PCR product, digested using *Bsi*WI, a restriction site introduced when designing the primers, was ligated on itself to create plasmid pSE103, which was sequenced to verify the presence of the introduced mutation. The 404 bp *Aat*II/*Eco*RI fragment from pSE103 was used to replace the corresponding wild-type fragment from plasmid pATPA2 (Drapier *et al.*, 1998), resulting in plasmid pSE203. Finally, a 5′ *petA–aadA* cassette (Choquet *et al.*, 2001) was inserted in reverse orientation with respect to the *atpA* gene into the unique *Bse*RI site of plasmid pSE203 to yield plasmid pK$atpA_{St}$.

Construction of plasmids containing a full-length or partial TDA1 *coding sequence.* Plasmid *pSNAN16* (Gumpel *et al.*, 1995), containing a 18 kb *Avr*II–*Nde*I fragment from cosmid cos11.B5 that encompasses the *MCA1* and *TDA1* genes, sub-cloned into the *Nde*I and *Spe*I sites of vector *pGEM5* (Promega, http://www.promega.com/), was digested with *Eco*RV and *Hin*dIII, blunt-ended using Klenow fragment, and re-ligated on itself to create plasmid *pTDA1-EH* that no longer contains the *MCA1* gene. *pTDA1-EH*, digested with *Nde*I and *Nhe*I and treated with Klenow fragment, was re-ligated on itself to create plasmid *pTDA1*, which contains a 6650 bp insert starting 550 bp upstream of the *TDA1* initiation codon and ending 601 bp downstream of the stop codon. *pTDA1* was then digested with *Sph*I and *Not*I or with *Not*I and *Spe*I, blunt-ended using Klenow fragment, and re-ligated on itself to create plasmids *pNter* or *pCter*, which contain *TDA1* coding sequences upstream or downstream, respectively, of the *Not*I site (Figure 8).

Sequencing of the *TDA1* cDNA

No *TDA1* ESTs were found in current libraries. Thus to amplify *TDA1* cDNAs, cultures of wild-type grown under various conditions [TAP or minimal medium, low (10 μE m^{-2} sec^{-1}) or medium (200 μE m^{-2} sec^{-1}) light conditions, exponential or early stationary phase) were pooled before RNA extraction. After DNase treatment, RNAs that had been further purified using a Qiagen RNeasy MinElute Cleanup Kit (http://www.qiagen.com/), according to the manufacturer's protocol, were reverse-transcribed using a TaKaRa™ reverse transcription kit (http://www.takara-bio.com/) and random primers. Partial and overlapping *TDA1* cDNAs, amplified using the primers listed in Table S1, were sequenced and assembled to generate the *TDA1* coding sequence.

Transformation experiments

Chloroplast transformations were performed using tungsten particle bombardment (Boynton and Gillham, 1993) as described by Kuras and Wollman (1994). Transformants were selected on TAP medium supplemented with spectinomycin (100 μg ml^{-1}) under low light (5–6 μE m^{-2} sec^{-1}) and sub-cloned in darkness on selective medium until they reached homoplasmy. Three independent transformants were analysed and proved identical. Correct insertion of transforming DNA and homoplasmy were checked by *Bsi*WI digestion of PCR products.

Nuclear transformations were performed by electroporation, as described by Shimogawara *et al.* (1998), using the following parameters: 25 μF/720 V cm^{-1}. Transformants were either selected for phototrophy under high light (200 μE m^{-2} sec^{-1}) on minimum medium (Harris, 1989) supplemented with 100 μg ml^{-1} ampicillin to prevent bacterial infection or for resistance to Zeocin (10 μg ml^{-1}) under low light. Plasmids *pTDA1, pCter, pNter* and *psh-MCA1* (Raynaud *et al.*, 2007) were linearized using *Sal*I, *Sal*I, *Not*I and *Xba*I, respectively, prior to transformation.

Purification of ribonucleic complexes on sucrose gradients

Polysomes were purified as described by Barkan (1993), Rott *et al.* (1998) and Minai *et al.* (2006). Figure S5a shows the sedimentation

profiles of wild-type chloroplast RNAs under $MgCl_2$ and EDTA conditions, and Figure S5b shows the sedimentation pattern of purified *Escherichia coli* 70S ribosomes under the same conditions. Although initial solutions were prepared at 15% or 55% w/v sucrose, the real sucrose concentration, determined by refractometry, ranged between 14% and 45%. Figure S5c shows the mean sucrose concentration for each fraction. If the sucrose concentration of fractions in a given experiment differed significantly from this distribution, the experiment was disregarded and repeated.

RNA isolation and analysis

RNA extraction and RNA blots were performed as described by Drapier *et al.* (1998) using probes derived from the coding sequences described by Eberhard *et al.* (2002). Scanning of ^{33}P RNA blots were done using a Typhoon scanner and the ImageQuant software package (GE Healthcare, http://www.gehealthcare.com/) .

Protein isolation and analysis

Pulse labelling, protein isolation, separation and immunoblot analysis were performed using exponentially growing cells (2×10^6 cells ml^{-1}) as described by Kuras and Wollman (1994) and Drapier *et al.* (2007). Cell extracts were loaded on an equal-chlorophyll basis.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Amino acid sequence and properties of the TDA1 protein.

Figure S2. Secondary structure prediction for the TDA1 protein.

Figure S3. Alignment of the TDA1 proteins of *Chlamydomonas reinhardtii* (Cr) and *Volvox carterii* (Vc).

Figure S4. Conserved residues between OPR repeats and RAP domains.

Figure S5. Characterization of the sedimentation profile of chloroplast transcripts in sucrose gradients.

Table S1. Oligonucleotides used in this study.

Table S2. OPR proteins in the nuclear genome of *Chlamydomonas reinhardtii*.

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REFERENCES

Anderson, P. and Kedersha, N. (2002a) Stressful initiations. *J. Cell Sci.* **115**, 3227–3234.

Anderson, P. and Kedersha, N. (2002b) Visibly stressed: the role of eIF2, TIA–1, and stress granules in protein translation. *Cell Stress Chaperones*, **7**, 213–221.

Anderson, P. and Kedersha, N. (2008) Stress granules: the Tao of RNA triage. *Trends Biochem. Sci.* **33**, 141–150.

Anderson, P. and Kedersha, N. (2009a) RNA granules: post-transcriptional and epigenetic modulators of gene expression. *Nat. Rev. Mol. Cell Biol.* **10**, 430–436.

Anderson, P. and Kedersha, N. (2009b) Stress granules. *Curr. Biol.* **19**, R397–R398.

Auchincloss, A.H., Zerges, W., Perron, K., Girard-Bascou, J. and Rochaix, J.D. (2002) Characterization of Tbc2, a nucleus-encoded factor specifically required for translation of the chloroplast *psbC* mRNA in *Chlamydomonas reinhardtii*. *J. Cell Biol.* **157**, 953–962.

Balczun, C., Bunse, A., Hahn, D., Bennoun, P., Nickelsen, J. and Kuck, U. (2005) Two adjacent nuclear genes are required for functional complementation of a chloroplast *trans*-splicing mutant from *Chlamydomonas reinhardtii*. *Plant J.* **43**, 636–648.

Barkan, A. (1993) Nuclear mutants of maize with defects in chloroplast polysome assembly have altered chloroplast RNA metabolism. *Plant Cell*, **5**, 389–402.

Barkan, A. and Goldschmidt-Clermont, M. (2000) Participation of nuclear genes in chloroplast gene expression. *Biochimie*, **82**, 559–572.

Blatch, G.L. and Lassle, M. (1999) The tetratricopeptide repeat: a structural motif mediating protein–protein interactions. *Bioessays*, **21**, 932–939.

Bolli, R., Mendiola-Morgenthaler, L. and Boschetti, A. (1981) Isolation and characterization of polysomes from thylakoid membranes of *Chlamydomonas reinhardii*. *Biochim. Biophys. Acta*, **653**, 276–287.

Boulouis, A., Raynaud, C., Bujaldon, S., Aznar, A., Wollman, F.A. and Choquet, Y. (2011) The nucleus-encoded *trans*-acting factor MCA1 plays a critical role in the regulation of cytochrome *f* synthesis in *Chlamydomonas* chloroplasts. *Plant Cell*, **23**, 333–349.

Boynton, J.E. and Gillham, N.W. (1993) Chloroplast transformation in *Chlamydomonas*. *Methods Enzymol.* **217**, 510–536.

Choquet, Y. and Wollman, F.A. (2002) Translational regulations as specific traits of chloroplast gene expression. *FEBS Lett.* **529**, 39–42.

Choquet, Y. and Wollman, F.A. (2009) The CES process. In *Chlamydomonas Source Book*, 2nd edn (Harris, E., Stern, D.B. and Whitman, G., eds). New York: Academic Press/Elsevier, pp. 1027–1064.

Choquet, Y., Stern, D.B., Wostrikoff, K., Kuras, R., Girard-Bascou, J. and Wollman, F.A. (1998) Translation of cytochrome *f* is autoregulated through the 5′ untranslated region of *petA* mRNA in *Chlamydomonas* chloroplasts. *Proc. Natl Acad. Sci. USA*, **95**, 4380–4385.

Choquet, Y., Wostrikoff, K., Rimbault, B., Zito, F., Girard-Bascou, J., Drapier, D. and Wollman, F.A. (2001) Assembly-controlled regulation of chloroplast gene translation. *Biochem. Soc. Trans.* **29**, 421–426.

Choquet, Y., Zito, F., Wostrikoff, K. and Wollman, F.A. (2003) Cytochrome *f* translation in *Chlamydomonas* chloroplast is autoregulated by its carboxyl-terminal domain. *Plant Cell*, **15**, 1443–1454.

Chua, N.H., Blobel, G., Siekevitz, P. and Palade, G.E. (1973) Attachment of chloroplast polysomes to thylakoid membranes in *Chlamydomonas reinhardtii*. *Proc. Natl Acad. Sci. USA*, **70**, 1554–1558.

Das, A.K., Cohen, P.W. and Barford, D. (1998) The structure of the tetratricopeptide repeats of protein phosphatase 5: implications for TPR-mediated protein–protein interactions. *EMBO J.* **17**, 1192–1199.

Dauvillee, D., Stampacchia, O., Girard-Bascou, J. and Rochaix, J.D. (2003) Tab 2 is a novel conserved RNA binding protein required for translation of the chloroplast *psaB* mRNA. *EMBO J.* **22**, 6378–6388.

Drapier, D., Girard-Bascou, J. and Wollman, F.A. (1992) Evidence for nuclear control of the expression of the *atpA* and *atpB* chloroplast genes in *Chlamydomonas*. *Plant Cell*, **4**, 283–295.

Drapier, D., Girard-Bascou, J., Stern, D.B. and Wollman, F.A. (2002) A dominant nuclear mutation in Chlamydomonas identifies a factor controlling chloroplast mRNA stability by acting on the coding region of the atpA transcript. *Plant J.* **31**, 687–697.

Drapier, D., Suzuki, H., Levy, H., Rimbault, B., Kindle, K.L., Stern, D.B. and Wollman, F.A. (1998) The chloroplast *atpA* gene cluster in *Chlamydomonas reinhardtii*. Functional analysis of a polycistronic transcription unit. *Plant Physiol.* **117**, 629–641.

Drapier, D., Rimbault, B., Vallon, O., Wollman, F.A. and Choquet, Y. (2007) Intertwined translational regulations set uneven stoichiometry of chloroplast ATP synthase subunits. *EMBO J.* **26**, 3581–3591.

Eberhard, S., Drapier, D. and Wollman, F.A. (2002) Searching limiting steps in the expression of chloroplast-encoded proteins: relations between gene copy number, transcription, transcript abundance and translation rate in the chloroplast of *Chlamydomonas reinhardtii*. *Plant J.* **31**, 149–160.

Emanuelsson, O. and von Heijne, G. (2001) Prediction of organellar targeting signals. *Biochim. Biophys. Acta*, **1541**, 114–119.

Esposito, D., Fey, J.P., Eberhard, S., Hicks, A.J. and Stern, D.B. (2003) *In vivo* evidence for the prokaryotic model of extended codon–anticodon interaction in translation initiation. *EMBO J.* **22**, 651–656.

Gumpel, N.J., Ralley, L., Girard-Bascou, J., Wollman, F.A., Nugent, J.H. and Purton, S. (1995) Nuclear mutants of *Chlamydomonas reinhardtii* defective in the biogenesis of the cytochrome b_6f complex. *Plant Mol. Biol.* **29**, 921–932.

Harris, E. (1989) *The Chlamydomonas Sourcebook. A Comprehensive Guide to Biology and Laboratory Use*. San Diego, CA: Academic Press.

Herrin, D.L. and Nickelsen, J. (2004) Chloroplast RNA processing and stability. *Photosynth. Res.* **82**, 301–314.

Hoglund, A., Blum, T., Brady, S., Donnes, P., Miguel, J.S., Rocheford, M., Kohlbacher, O. and Shatkay, H. (2006) Significantly improved prediction of subcellular localization by integrating text and protein sequence data. *Pac. Symp. Biocomput.* **11**, 16–27.

Hoober, J.K. and Blobel, G. (1969) Characterization of the chloroplastic and cytoplasmic ribosomes of *Chlamydomonas reinhardii*. *J. Mol. Biol.* **41**, 121–138.

Horton, P., Park, K.J., Obayashi, T., Fujita, N., Harada, H., Adams-Collier, C.J. and Nakai, K. (2007) WoLF PSORT: protein localization predictor. *Nucleic Acids Res.* **35**, W585–W587.

Johnson, X., Wostrikoff, K., Finazzi, G., Kuras, R., Schwarz, C., Bujaldon, S., Nickelsen, J., Stern, D.B., Wollman, F.A. and Vallon, O. (2010) MRL1, a conserved pentatricopeptide repeat protein, is required for stabilization of *rbcL* mRNA in *Chlamydomonas* and *Arabidopsis*. *Plant Cell*, **22**, 234–248.

Kedersha, N., Cho, M.R., Li, W., Yacono, P.W., Chen, S., Gilks, N., Golan, D.E. and Anderson, P. (2000) Dynamic shuttling of TIA-1 accompanies the recruitment of mRNA to mammalian stress granules. *J. Cell Biol.* **151**, 1257–1268.

Kedersha, N., Stoecklin, G., Ayodele, M., Yacono, P., Lykke-Andersen, J., Fritzler, M.J., Scheuner, D., Kaufman, R.J., Golan, D.E. and Anderson, P. (2005) Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. *J. Cell Biol.* **169**, 871–884.

Klinkert, B., Schwarz, C., Pohlmann, S., Pierre, Y., Girard-Bascou, J. and Nickelsen, J. (2005) Relationship between mRNA levels and protein accumulation in a chloroplast promoter-mutant of *Chlamydomonas reinhardtii*. *Mol. Genet. Genomics*, **274**, 637–643.

Kuras, R. and Wollman, F.A. (1994) The assembly of cytochrome b_6f complexes: an approach using genetic transformation of the green alga *Chlamydomonas reinhardtii*. *EMBO J.* **13**, 1019–1027.

Lee, I. and Hong, W. (2004) RAP – a putative RNA-binding domain. *Trends Biochem. Sci.* **29**, 567–570.

Lemaire, C. and Wollman, F.A. (1989) The chloroplast ATP synthase in *Chlamydomonas reinhardtii* II. Biochemical studies on its biogenesis using mutants defective in photophosphorylation. *J. Biol. Chem.* **264**, 10235–10242.

Loiselay, C., Gumpel, N.J., Girard-Bascou, J., Watson, A.T., Purton, S., Wollman, F.A. and Choquet, Y. (2008) Molecular identification and function of *cis*- and *trans*-acting determinants for *petA* transcript stability in *Chlamydomonas reinhardtii* chloroplasts. *Mol. Cell. Biol.* **28**, 5529–5542.

Merendino, L., Perron, K., Rahire, M., Howald, I., Rochaix, J.D. and Goldschmidt-Clermont, M. (2006) A novel multifunctional factor involved in *trans*-splicing of chloroplast introns in *Chlamydomonas*. *Nucleic Acids Res.* **34**, 262–274.

Minai, L., Wostrikoff, K., Wollman, F.A. and Choquet, Y. (2006) Chloroplast biogenesis of photosystem II cores involves a series of assembly-controlled steps that regulate translation. *Plant Cell*, **18**, 159–175.

Nickelsen, J., van Dillewijn, J., Rahire, M. and Rochaix, J.D. (1994) Determinants for stability of the chloroplast *psbD* RNA are located within its short leader region in *Chlamydomonas reinhardtii*. *EMBO J.* **13**, 3182–3191.

Raynaud, C., Loiselay, C., Wostrikoff, K., Kuras, R., Girard-Bascou, J., Wollman, F.A. and Choquet, Y. (2007) Evidence for regulatory function of nucleus-encoded factors on mRNA stabilization and translation in the chloroplast. *Proc. Natl Acad. Sci. USA*, **104**, 9093–9098.

Rochaix, J.D. (2004) Genetics of the biogenesis and dynamics of the photosynthetic machinery in eukaryotes. *Plant Cell*, **16**, 1650–1660.

Rott, R., Levy, H., Drager, R.G., Stern, D.B. and Schuster, G. (1998) 3′-processed mRNA is preferentially translated in *Chlamydomonas reinhardtii* chloroplasts. *Mol. Cell. Biol.* **18**, 4605–4611.

Rymarquis, L.A., Handley, J.M., Thomas, M. and Stern, D.B. (2005) Beyond complementation. Map-based cloning in *Chlamydomonas reinhardtii*. *Plant Physiol.* **137**, 557–566.

Sane, A.P., Stein, B. and Westhoff, P. (2005) The nuclear gene *HCF107* encodes a membrane-associated R-TPR (RNA tetratricopeptide repeat)-containing protein involved in expression of the plastidial *psbH* gene in Arabidopsis. *Plant J.* **42**, 720–730.

Sasarman, F., Brunel-Guitton, C., Antonicka, H., Wai, T. and Shoubridge, E.A. (2010) LRPPRC and SLIRP interact in a ribonucleoprotein complex that regulates posttranscriptional gene expression in mitochondria. *Mol. Biol. Cell*, **21**, 1315–1323.

Schmitz-Linneweber, C. and Small, I. (2008) Pentatricopeptide repeat proteins: a socket set for organelle gene expression. *Trends Plant Sci.* **13**, 663–670.

Schwarz, C., Elles, I., Kortmann, J., Piotrowski, M. and Nickelsen, J. (2007) Synthesis of the D2 protein of photosystem II in *Chlamydomonas* is controlled by a high molecular mass complex containing the RNA stabilization factor Nac2 and the translational activator RBP40. *Plant Cell*, **19**, 3627–3639.

Shimogawara, K., Fujiwara, S., Grossman, A. and Usuda, H. (1998) High-efficiency transformation of *Chlamydomonas reinhardtii* by electroporation. *Genetics*, **148**, 1821–1828.

Simarro, M., Gimenez-Cassina, A., Kedersha, N. *et al.* (2010) Fast kinase domain-containing protein 3 is a mitochondrial protein essential for cellular respiration. *Biochem. Biophys. Res. Commun.* **401**, 440–446.

Small, I., Peeters, N., Legeai, F. and Lurin, C. (2004) Predotar: a tool for rapidly screening proteomes for N-terminal targeting sequences. *Proteomics*, **4**, 1581–1590.

Stevens, D.R., Rochaix, J.D. and Purton, S. (1996) The bacterial phleomycin resistance gene *ble* as a dominant selectable marker in *Chlamydomonas*. *Mol. Gen. Genet.* **251**, 23–30.

Tian, Q., Taupin, J., Elledge, S., Robertson, M. and Anderson, P. (1995) Fas-activated serine/threonine kinase (FAST) phosphorylates TIA-1 during Fas-mediated apoptosis. *J. Exp. Med.* **182**, 865–874.

Uniacke, J. and Zerges, W. (2008) Stress induces the assembly of RNA granules in the chloroplast of *Chlamydomonas reinhardtii*. *J. Cell Biol.* **182**, 641–646.

Vaistij, F.E., Boudreau, E., Lemaire, S.D., Goldschmidt-Clermont, M. and Rochaix, J.D. (2000a) Characterization of Mbb1, a nucleus-encoded tetratricopeptide-like repeat protein required for expression of the chloroplast *psbB/psbT/psbH* gene cluster in *Chlamydomonas reinhardtii*. *Proc. Natl Acad. Sci. USA*, **97**, 14813–14818.

Vaistij, F.E., Goldschmidt-Clermont, M., Wostrikoff, K. and Rochaix, J.D. (2000b) Stability determinants in the chloroplast *psbB/T/H* mRNAs of *Chlamydomonas reinhardtii*. *Plant J.* **21**, 469–482.

Weber, M.J. and DeMoss, J.A. (1966) The inhibition by chloramphenicol of nascent protein formation in *E. coli*. *Proc. Natl Acad. Sci. USA*, **55**, 1224–1230.

Wollman, F.A., Minai, L. and Nechushtai, R. (1999) The biogenesis and assembly of photosynthetic proteins in thylakoid membranes. *Biochem. Biophys. Acta*, **1411**, 21–85.

Wostrikoff, K., Choquet, Y., Wollman, F. and Girard-Bascou, J. (2001) TCA1, a single nuclear-encoded translational activator specific for *petA* mRNA in *Chlamydomonas reinhardtii* chloroplast. *Genetics*, **159**, 119–132.

Xaplanteri, M.A., Andreou, A., Dinos, G.P. and Kalpaxis, D.L. (2003) Effect of polyamines on the inhibition of peptidyltransferase by antibiotics: revisiting the mechanism of chloramphenicol action. *Nucleic Acids Res.* **31**, 5074–5083.